CYCLE DEPENDENT VARIATION OF X-RAY EFFECTS ON SYNCHRONOUS MITOSIS AND THYMIDINE KINASE INDUCTION IN *PHYSARUM POLYCEPHALUM* *

W.SACHSENMAIER, E.BOHNERT, B.CLAUSNIZER

Institut für Biochemie und experimentelle Krebsforschung der Universität Innsbruck, Austria, and Institut für experimentelle Krebsforschung am Deutschen Krebsforschungszentrum Heidelberg, Germany,

and

O.F.NYGAARD

Division of Radiation Biology, Department of Radiology, Case Western Reserve University, Cleveland, Ohio, USA

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1. Introduction

Mitoses in the multinuclear plasmodium of the myxomycete *Physarum polycephalum* occur spontaneously synchronous [1]. Thymidine kinase (EC 2.7.1.21) increases periodically just prior to and during each mitosis [2, 3] suggesting that the regulation of this enzyme is closely linked to the control of the mitotic cycle in *Physarum*. In order to obtain further information about the mechanism controlling thymidine kinase induction and its correlation with mitosis, the effect of ionizing radiation on the timing of these two events was studied. Since X-rays exert a retarding effect on mitosis in many systems without affecting overall cell growth [4], they appear to act rather specifically on the control of the division cycle. The action mechanism, however, is still obscure. The question was asked whether a radiation induced delay of mitosis is accompanied by a corresponding change of the time marker for thymidine kinase induction or whether these two events can be dissociated. Further, the cycle dependent variation of the X-ray effects was studied in order to learn more about the possible action mechanism of ionizing radiation. The results indicate that the radiosensitivity of *Physarum* plasmodia changes drastically during the synchronous nuclear division cycle. The time markers of mitosis and of thymidine kinase induction are equally affected by X-rays suggesting that the control mechanisms of these two events are indeed closely related or even identical.

2. Methods

Disc shaped macroplasmodia of *Physarum polycephalum* with a diameter of 4 to 6 cm were cultivated on a sterile liquid medium as described [5, 6]. Entire plasmodia or segments were irradiated with a Siemens "Dermophan" X-ray generator (29 kV, 25 mA, 0.3 mm Al filter, dose rate 870 r/min). Mitotic stages were observed in ethanol fixed smears with a phase contrast microscope [7]. Thymidine kinase activity was determined in the high speed supernatant of plasmodial homogenates as described [2, 3].

3. Results and discussion

3.1. *Cycle dependent variation of mitotic delay*

Previous studies by Nygaard et al. have shown that X-irradiation of *Physarum* with 9000 r retards mitosis most effectively when applied during early interphase (S-period) or at the end of the G_2-period [9]. Following up these earlier studies in more detail

* Dedicated to Prof.Dr.K.H.Bauer on his 80th birthday.

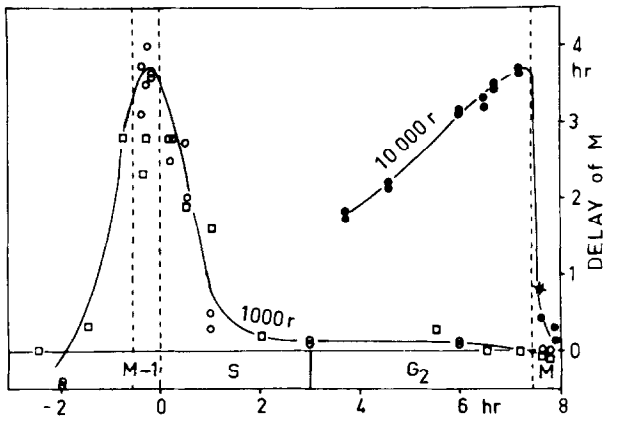

Fig.1. Variation of radiosensitivity during the nuclear division cycle. Macroplasmodia were cut into 4 equal segments about 3 hr prior to the expected second synchronous mitosis. Individual segments were removed from the medium at various time intervals, irradiated in air with 1000 r or 10,000 r, and returned onto nutrient medium. Each point represents the delay of mitosis (M) relative to an untreated control. The results of three separate experiments are marked with different symbols. (M), (M-1): synchronous mitoses (prophase to telophase); (S): period of nuclear DNA synthesis; (G_2): premitotic gap: there is no G_1-period in the nuclear cycle of this organism [20, 21]. (*): a few pycnotic nuclei were observed occasionally after irradiation in early prophase.

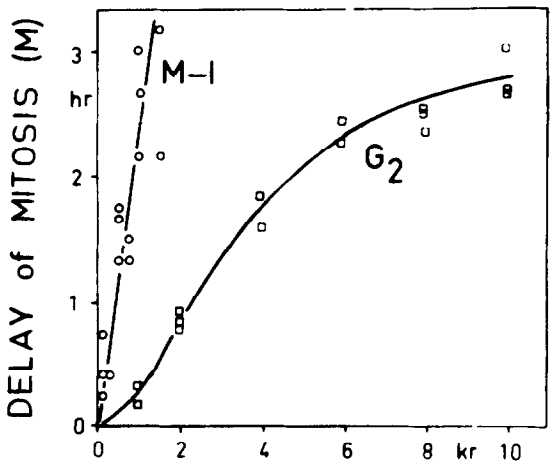

Fig. 2. Dose dependent delay of mitosis (M) in plasmodia irradiated during the preceding metaphase (M-1) or in G_2-period (1.7 hr prior to mitosis M).

we find (fig. 1) that irradiation with a lower dose (1000 r) reveals only one period in the cycle with high radiosensitivity. The peak coincides with mitosis (M-1) which precedes the delayed mitosis (M). The declining part of the peak extends into early S-period but little effect is seen during the rest of the cycle. With a 10 times higher dose (10,000 r), however, a second sensitive period is observed during late interphase prior to mitosis (M). The effect increases steadily as the time of irradiation approaches the end of the G_2-period and then drops sharply at the time of the onset of prophase. The dose dependent increase of the effect is almost linear up to at least 1,500 r if the irradiation occurs during mitosis (M-1) but follows a slightly sigmoidal pattern during the less sensitive G_2-period (fig. 2).

The results suggest that X-rays interfere with the mitotic cycle by different mechanisms depending on the position of the cycle at the time of treatment. The period of maximal radiosensitivity overlaps but does not exactly coincide with the period of nuclear DNA replication (S-phase). It is unlikely therefore that the radiosensitive molecular event is identical with overall nuclear DNA synthesis. This is supported by the finding that irradiation with 1000 r does not significantly alter the rate of ^{14}C-thymidine incorporation into DNA during the first S-period (fig. 3). Labeling of DNA during the second S-period, which is delayed

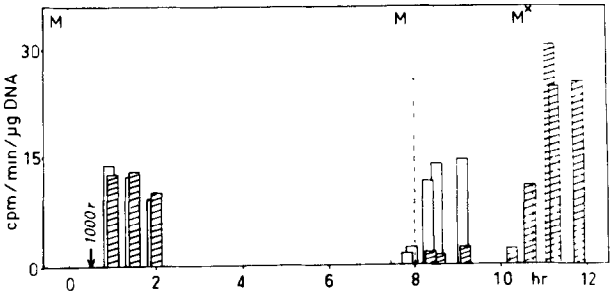

Fig. 3. Specific radioactivity of DNA after pulse labeling with ^{14}C-thymidine. Four replicate macroplasmodia were divided into halves at the time of mitosis (III). One moiety of each culture was irradiated with 1000 r 30 min after telophase (early S-period) the second moiety served as a control. Small pieces V$\sim$2 cm^2 were dissected from treated and control plasmodia at various time intervals, incubated 15 min on medium containing 0.05 µCi/ml ^{14}C-thymidine (30 mCi/mmole), immersed into 2 ml cold 0.25 N trichloroacetic acid (TCA) and processed as described [21]. The acid-insoluble fraction was then extracted with 1 ml hot TCA (15 min, 70°) and the supernatant was analyzed for DNA (8) and radioactivity (scintillation counter). Columns indicate specific radioactivity of DNA in control (white) and treated (shaded) segments. (M), (M^x): mitosis in normal and irradiated plasmodia respectively.

parallel to the delay of the next mitosis, even exceeds the control rate. The latter effect most likely results from the increased production of thymidine kinase as discussed below (3.2).

The high radiosensitivity prior to the onset of the S-period may nevertheless be causally related to the process of DNA replication. This as also suggested by recent observations of Nygaard et al. [10] that the period of high radiosensitivity in *Physarum* can be prolonged by inhibiting DNA synthesis with 5-fluorodeoxyuridine. The position of the peak of radiosensitivity would agree with the idea that the target is DNA and the primary damage is immediately "fixed" by the subsequent replication of the DNA molecules; i.e. a single strand lesion is by-passed by the replicating fork leaving a gap in the newly synthesized strand which cannot be closed rapidly. The ultimate cause of the retarding effect on mitosis thus may be a disturbance of the transcription at these defective gene sites. On the other hand, a similar damage inflicted upon nonreplicating DNA would quickly be repaired, explaining the much lower radiosensitivity during the G_2-period. The relative radioresistance of the second half of the S-period could mean that early replicating genes are more important for the control of mitosis than later replicating genes.

A different mechanism seems to be responsible for the retardation of mitosis by large X-ray doses applied during the late G_2-period. Perhaps double strand breaks of DNA are involved which require much higher radiation energies to be formed and are less efficiently repaired than single strand breaks [11]. On the other hand, a DNA-protein complex [12] or components other than DNA may be considered as the primary target as well. The steady increase of radiosensitivity toward the end of the G_2-period suggests that the radiation damage is repaired with a half time of about 3 hr (fig. 1). There is some evidence from studies with other systems that the recovery from radiation damage leading to mitotic delay requires protein synthesis but not RNA synthesis [13–15]. This could mean that ionizing radiation interferes directly with proteins essential for mitosis or indirectly by affecting their synthesis at the translation level. While such a mechanism seems less suitable to explain the close correlation of the early sensitive period with DNA synthesis, it may well be responsible for the antimitotic effect of radiation in the late

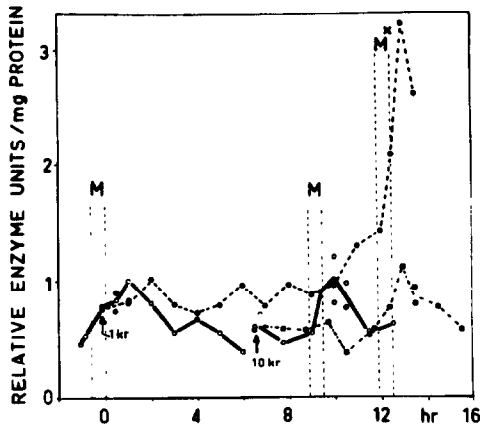

Fig. 4. Cycle dependent fluctuation of thymidine kinase in normal and irradiated plasmodia. A series of 6 replicate macroplasmodia was prepared. Each culture was divided into halves at the time of mitosis (III) and one moiety irradiated with 1000 r immediately. A different set of cultures was treated similary with 10,000 r 2 hr prior to the expected onset of mitosis. Quarter segments (~5 cm^2) were harvested at various time intervals, homogenized in 1.5 ml tris buffer (0.05 M, pH 7.5) + 0.002 M mercaptoethanol with a Branson sonicator (3 x 2 sec stage 3) and centrifuged (30 min 120,000 *g*). Enzyme activity was assayed in a mixture containing 10 mM ATP, 10 mM 3-phosphoglycerate, 10 mM $MgCl_2$, 40 mM tris-HCl pH 7.5, 2.5 mM EDTA-Na. 1 mM mercaptoethanol, 8 μM ^{14}C-thymidine (59 μCi/mmole), plasmodial extract 0.5–1.0 mg protein/ml; total volume 0.2 ml. Aliquots (25 μl) were pipetted onto pieces of anion exchange paper (Whatman DE 81, 1.6 X 2 cm) after 0 and 20 min incubation at 27° and processed as described [2]. The amount of ^{14}C-thymidine phosphorylated and retained by the paper was measured with a scintillation counter. o——o: controls; •– – –•, ■–·–■: irradiated with 1000 r and 10,000 r respectively; (M), (M^x): mitosis in controls and irradiated plasmodia respectively.

G_2-phase. This would agree very well with our observation that the end of the radiosensitive period in G_2-phase exactly coincides with the last point of the cycle where inhibition of protein synthesis with cycloheximide prevents the onset of mitosis [3, 16].

3.2. *Effect on thymidine kinase production.*

Irradiation with 1000 r during mitosis (M-1) does not immediately affect the pattern of thymidine kinase activity (fig. 4) but later on the enzyme level stays somewhat higher than in the controls. At the time of the delayed mitosis (M^x) the enzyme sharply

increases, reaching a peak far above the control maximum. The time marker of thymidine kinase induction therefore is shifted concomitantly with mitosis but the yield of enzyme is largely enhanced. Irradiation with 10,000 r during late G_2-period causes the same delay of mitosis (~3 hr) as irradiation with 1000 r during metaphase (M-1). Again, the induction of the enzyme is delayed parallel to the delay of mitosis (M), however, the yield does not exceed the control peak.

These results indicate that X-irradiation equally retards the onset of mitosis and the periodic induction of thymidine kinase suggesting that the timing mechanisms of these events are closely interrelated. As a working hypothesis we assume that the nuclear division cycle is controlled by the sequential transcription of a particular set of genes including the gene of thymidine kinase. Progress along this sequence requires concomitant synthesis of RNA and protein [3, 17, 18]. Thus, a particular gene of this sequence may be transcribed only after the gene product of the preceding step has been formed [19]. A delay of the transcription of the thymidine kinase gene, which presumably occurs during late G_2-period [3], may result from an interaction with any preceding step at the transcription or translation level. Based on our data we favor the idea that irradiation during the early sensitive period delays the induction of thymidine kinase as well as the onset of mitosis by an interference with the transcription of "early" genes of the sequence, whereas irradiation during G_2-phase may affect the function of "late" genes at the translation level.

The remarkable stimulation of enzyme production which occurs only after irradiation during the early sensitive period could be interpreted by the following hypothesis. The yield of enzyme synthesis during the induction period may normally be restricted by a repressor which acts at the transcription or translation level. Formation of this factor is supposed to be controlled by a gene which is active only during the early part of the cycle. Assuming that the repressor is fairly stable, its level should gradually decrease during the interphase due to dilution but not completely disappear by the time when the next pulse of enzyme production is induced at the end of the G_2-period. Hence, the enzyme forming system normally does not operate with its full capacity. Irradiation during mitosis or early S-period may inactivate the repressor gene and thus prevent a new pulse of inhibitor production. The repressor level will further decrease and even disappear completely, permitting unrestricted enzyme production when the thymidine kinase gene is turned on at the end of the prolonged G_2-period. Contrary to this, irradiation during G_2-phase does not affect the repressor which has been formed already prior to the treatment; thus, the yield of enzyme production is normal.

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